APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1983, p. 1134–1139
0099-2240/83/111134-06$02.00/0

Vol. 46, No. 5

Susceptibility of *Legionella pneumophila* to Chlorine in Tap Water

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Received 24 March 1983/Accepted 9 August 1983

A study was conducted to compare the susceptibility of legionellae and coliforms to disinfection by chlorine. The chlorine residuals used were similar to concentrations that might be found in the distribution systems of large public potable water supplies. The effects of various chlorine concentrations, temperatures, and pH levels were considered. A number of different *Legionella* strains, both environmental and clinical, were tested. The results indicate that legionellae are much more resistant to chlorine than are coliform bacteria. At 21°C, pH 7.6, and 0.1 mg of free chlorine residual per liter, a 99% kill of *L. pneumophila* was achieved within 40 min, compared with less than 1 min for *Escherichia coli*. The observed resistance is enhanced as conditions for disinfection become less optimal. The required contact time for the removal of *L. pneumophilia* was twice as long at 4°C than it was at 21°C. These data suggest that legionellae can survive low levels of chlorine for relatively long periods of time.

During the past several years, *Legionella pneumophila* has been isolated from shower heads, taps, mixing valves, and hot water tanks of hospitals, hotels, and homes (7, 8, 25–27, 29). In a number of cases, the occurrence of legionellae in the plumbing systems was associated with disease; in other cases, it was not.

These bacteria have been found primarily in hot water systems. In particular, large numbers of legionellae have been detected in the sediment that accumulates at the bottom of institutional hot water tanks. Typically, the temperature at the bottom of the tanks, especially in hospital tanks intentionally maintained at relatively low temperatures (e.g., 43 to 55°C), falls within the optimal range for the growth of these organisms (19, 29). It has been shown experimentally that *L. pneumophila* grows in unsterilized tap water within the range of the temperatures found at the bottom of institutional tanks (31). This observation led to the hypothesis that hot water tanks act as breeding sites for the contamination of plumbing systems (29).

A question arises concerning the initial introduction of *L. pneumophila* into the hot water tanks. It has been suggested that plumbing systems may be seeded by small numbers of legionellae from public water supply reservoirs (25, 29). However, attempts to actually isolate these bacteria from the mains of water supplies have not been successful (12). Such evidence would be difficult to obtain since the legionellae may occur sporadically and in low numbers.

Legionellae in a public water supply would be exposed to chlorine concentrations that had been adjusted to control the presence of the indicator coliform bacteria. A number of studies have been conducted to determine the bactericidal effectiveness of a variety of disinfectants against *L. pneumophila* (11, 13, 15). Most of this work has been directed toward problem areas such as cooling towers and evaporative condensers of air conditioning systems. Skaliy et al. (24) found that free chlorine at concentrations of 3.3 and 6.6 mg/liter rapidly inactivated *L. pneumophila*. These relatively high chlorine concentrations were typical of those utilized in cooling towers. Wang et al. (30) examined the effectiveness of disinfectants at concentrations normally used in hospitals for the decontamination of tissues and surfaces. The investigation included the effect of relatively high concentrations of hypochlorite on both *L. pneumophila* and *Escherichia coli*. Their data suggested that legionellae might be somewhat more resistant to these high chlorine concentrations than are the coliform bacteria. They also raised the suspicion that the amount of residual chlorine recommended for standard water purification might not be sufficient for killing *L. pneumophila* when the bacteria are present in high numbers.

Our study pursued the question of *Legionella*

susceptibility to chlorine by examining the bactericidal effectiveness of chlorine at levels which might be found in public water distribution systems. A number of *Legionella* strains from several sources, both environmental and clinical, were examined for susceptibility to chlorine. A comparison was made with *E. coli, Klebsiella pneumoniae*, and *Enterobacter aerogenes* since these bacteria are members of the coliform group which is the commonly accepted microbial indicator for disinfection. Consideration was also given to measuring changes in susceptibility of *L. pneumophilia* to variations in chlorine concentration, temperature, and pH level that might be found in different water systems.

MATERIALS AND METHODS

Bacteria. A number of bacterial strains from various sources were used in this study (Table 1). Several environmental strains of *L. pneumophila* were isolated from samples collected from the Allegheny River in Pittsburgh, Pa. This river is the source of water for the municipal water supply system. To obtain these isolates, 20 liters of river water were concentrated to 10 ml by centrifugation on a Sorvall model RC-2B centrifuge that was equipped with a continuous-flow attachment. Due to the biological complexity of the river water, acid and heat enrichment procedures were used to exclude competing microorganisms. The concentrate was heated for 30 min at 50°C and then treated with 2 parts of a 0.2 M HCl–0.2 M KCl buffer solution (pH 2.2) (3, 31). The sample was then plated on a selective medium, differential glycine-vancomycin-polymyxin B agar (28). *L. pneumophilia* was identified on the basis of colonial morphology, the inability to grow on unsupplemented buffered charcoal-yeast extract agar, and the direct immunofluorescence test (6, 28). The environmental isolates used in this study had been subcultured three times on artificial medium before this experiment.

Environmental strains of *L. pneumophila* serogroups 1 and 6 and *Legionella micdadei* were also isolated from water and sediment which had been collected from the bottom of hospital hot water tanks by direct plating of the samples on differential glycine-vancomycin-polymyxin B agar.

The Centers for Disease Control-derived strain of *L. pneumophila* (Philadelphia 1), the clinical isolate of *L. micdadei* (EK), and the American Type Culture Collection-derived strains of *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were kindly supplied by A. W. Pasculle of the Presbyterian-University Hospital, Pittsburgh, Pa.

Experimental procedure. The bactericidal effectiveness of chlorine was examined by inoculating tap water with known quantities of legionellae and treating these aquatic test systems with chlorine. The action of chlorine was stopped by the addition of 0.1 ml of a 10% (wt/vol) solution of sodium thiosulfate to a 10-ml sample. Viable counts of legionellae were obtained by plating both 0.1 and 0.5 ml of a test system on buffered charcoal-yeast extract agar (21). Colony counts were performed after incubation of the plates at 37°C for 7 days. Appropriate chlorine, bacteria, and thiosulfate controls were included in each experiment. The inhibition of *L. pneumophila* and the other bacteria by sodium thiosulfate was tested by the addition of the thiosulfate solution to test systems in the presence and absence of chlorine. The exposure of the bacteria in these test systems to the thiosulfate for up to 2 h did not affect their viability compared with control samples which did not contain sodium thiosulfate.

TABLE 1. Bacteria tested for chlorine resistance

Bacteria	Origin (strain)[a]
L. pneumophila	
Serogroup 1	Allegheny River
Serogroup 1	CDC (Philadelphia 1)
Serogroup 1	Hospital hot water tank
Serogroup 6	Hospital hot water tank
L. micdadei	Hospital hot water tank
	Clinical specimen (EK)
E. coli	ATCC (ATCC 25922)
	Allegheny River
S. aureus	ATCC (ATCC 25923)
K. pneumoniae	Allegheny River
E. aerogenes	Clinical specimen

[a] CDC, Centers for Disease Control; ATCC, American Type Culture Collection.

The basic experiments involved a comparison of an environmental isolate of *L. pneumophila* serogroup 1 from the Allegheny River with an American Type Culture Collection-derived strain of *E. coli*. Both bacteria were exposed to identical chlorine concentrations under the same environmental conditions. A free chlorine residual of 0.1 mg/liter was used as the "standard" chlorine concentration. The standard environmental conditions for the basic experiments consisted of pH 7.6 at 21°C. After the addition of chlorine, the sample was rapidly stirred for 30 s at 200 rpm with a Teflon-coated magnetic stirring bar and then slowly stirred (60 rpm) for the remainder of the experiment. The above chlorine concentration and environmental conditions were chosen to simulate conditions that might be found in the distribution of a large public water supply.

In addition to performing experiments under standard conditions, the comparison between *L. pneumophila* and *E. coli* was extended to other chlorine residuals and environmental conditions. In studying the effects of different chlorine concentrations, the same experiment was repeated under standard conditions of pH and temperature but at free chlorine residuals of 0.2 and 0.5 mg/liter. Temperature variations, 4 and 32°C, were tested with a standard chlorine residual of 0.1 mg/liter and pH 7.6. Similarly, the effect of pH 6.0, 7.0, and 7.6 was determined under standard conditions of 0.1 mg of total chlorine per liter at 21°C.

Test system and chlorine determination. The aquatic test system consisted of sterile 1-liter Erlenmeyer flasks containing 600 ml of tap water. The water was obtained from a tap in the municipal water distribution system. Tap water was used because the purpose of

TABLE 2. Comparison of chlorine demand of boiled tap water with demand of deionized, distilled water[a]

Boiled tap water		Deionized distilled water	
Total chlorine[b]	Free chlorine[b]	Total chlorine[b]	Free chlorine[b]
0.05	0.05	0.05	0.05
0.10	0.10	0.10	0.10
0.25	0.25	0.25	0.20
0.35	0.35	0.35	0.30
0.50	0.50	0.50	0.45
0.60	0.60	0.60	0.55

[a] Essentially chlorine demand-free.
[b] Milligrams per liter as determined by the amperometric method.

this study was to investigate the survival of legionellae in a municipal water system. Dechlorination of the tap water was accomplished by boiling it before use. The water was then buffered with a phosphate buffer. KH_2PO_4 (0.5 M) and K_2HPO_4 (0.5 M) were mixed and diluted to a final pH of 6.0, 7.0, or 7.6 (standard) and a final concentration of 10 mM. A 100-mg/liter stock chlorine solution was prepared by dissolving calcium hypochlorite in sterile, distilled, deionized water. A Milli-Q system (Millipore Corp., Bedford, Mass.) was used to deionize the water. Chlorination of the test system was achieved by adding precalculated volumes of this stock to the buffered tap water. Free and total chlorine concentrations were measured at the beginning and end of each experiment by the amperometric method (2) to ensure that no unexpected chlorine demand had appeared in the test system water. Free and total chlorine measurements were also performed at the end of each experiment to determine the degree of chlorine depletion. Chlorine loss never exceeded 10% during any of the experiments.

Initially, the chlorine demand of boiled tap water was compared with that of essentially demand-free, distilled, deionized water. Various amounts of hypochlorite were added to portions of each type of water, and the total and free chlorine concentrations were measured. Boiled tap water was found to be essentially demand-free (Table 2).

To prepare inocula for the test system, *Legionella* and non-*Legionella* bacteria were cultured on buffered charcoal-yeast extract agar at 37°C. Legionellae were incubated for 76 h, and the non-*Legionella* bacteria were incubated for 24 h. The bacteria were scraped from the plate, washed twice with 30 ml of distilled water, and then suspended in 5 ml of distilled water. This inoculum was added to the aquatic test system to achieve a bacterial density of ca. 3,000 CFU/ml. This density of *L. pneumophila* is within the range reported in contaminated hot water tanks (29).

RESULTS

The effect of chlorine on *L. pneumophila* at various concentrations of chlorine, contact times, pH levels, and temperatures is summarized in Fig. 1 to 3. The results are expressed in terms of percent survival at progressively longer

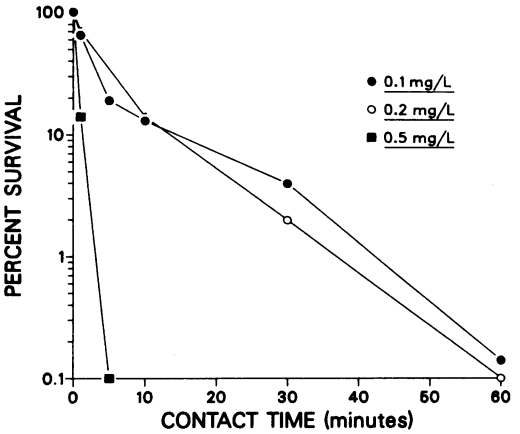

FIG. 1. Bactericidal effect of different concentrations of chlorine on *L. pneumophila* in tap water at pH 7.6 and 21°C.

times of exposure under each of the sets of conditions. *E. coli* was not detected in the samples within min 1 of treatment with chlorine. Identical results were obtained with *S. aureus* as well as with a strain of *K. pneumoniae* that had been isolated from a sample of river water. A river water sample containing a natural population of coliforms was also tested. These coliform bacteria were likewise killed within min 1 of treatment. Because the earliest sampling period after the addition of chlorine was 1 min, bacteria other than *L. pneumophilia* are not represented in the figures.

Under the standard conditions of pH 7.6, a temperature of 21°C, and a free chlorine residual of 0.1 mg/liter, a 99% kill of the legionellae did not occur until a contact time of between 30 and

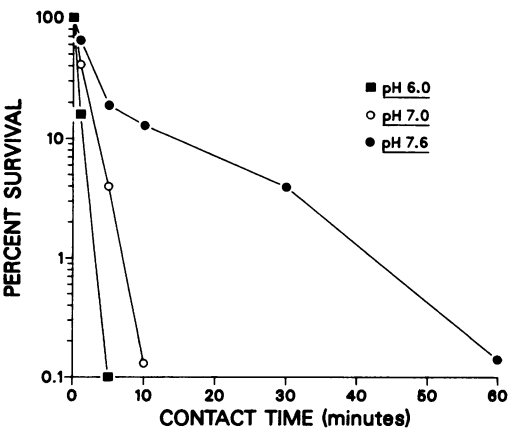

FIG. 2. Effect of pH on bactericidal activity of 0.1 mg of chlorine per liter on *L. pneumophila* in tap water at 21°C.

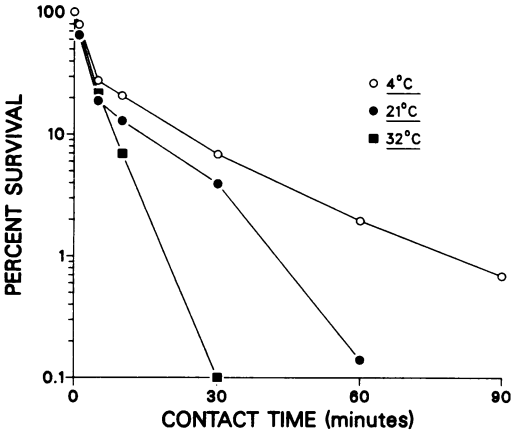

FIG. 3. Effect of temperature on bactericidal activity of 0.1 mg of chlorine per liter on *L. pneumophila* in tap water at pH 7.6.

60 min had elapsed. In addition to the standard bacterial concentration of 3,000 CFU/ml, a 10-fold increase and a 10-fold decrease in the number of bacteria were also tested. The kill rate was not affected by these changes. This latter finding is consistent with the observations of Butterfield et al. on other bacterial species (5). Increasing the total chlorine concentration (Fig. 1) predictably enhanced the bactericidal effect, resulting in a 99% kill within the first 5 min at a concentration of 0.5 mg/liter.

Decreasing the pH exerted an effect similar to that of increasing the chlorine concentration (Fig. 2). A contact time of ca. 40 min was required to eliminate 99% of the *Legionella* population at pH 7.6. In contrast, less than 10 min was required at pH 7.0 and less than 5 min was required at pH 6.0.

Temperature also exerted a dramatic influence on the chlorine disinfection of *L. pneumophilia* (Fig. 3). The time required for a 99% kill at 0.1 mg of chlorine per liter decreased from 40 min at room temperature to less than 30 min at the higher temperature of 32°C. At 4°C, between 60 and 90 min was required for a 99% kill.

In addition to examining the bactericidal effectiveness of chlorine on a strain of *L. pneumophila* that had been isolated from a river water sample, a number of other environmental and clinical isolates of legionellae were tested (Table 3). All of these isolates were studied under the standard conditions of 0.1 mg chlorine per ml, pH 7.6, and a temperature of 21°C. The contact times necessary to eliminate 99% of these populations were as long or longer than those required for the river isolate of *L. pneumophila* that had been used as the primary test organism. Long contact times were required for the clinical and environmental isolates of *L. pneumophila*, regardless of serogroup or origin, as well as for *L. micdadei*. These results indicate that legionellae can survive for relatively long periods of time at low concentrations of chlorine under a variety of temperatures and levels of pH.

DISCUSSION

Hypochlorites have been employed for the disinfection of water for potable use since 1894 (22). The basis for the establishment of effective levels of chlorine is the susceptibility of *E. coli* and other coliform bacteria. These bacteria have served as indicators of the bacteriological quality of water supplies since the publication of the first edition of *Standard Methods of Water Analysis* in 1905 (1). Some waterborne pathogens have been shown to be more resistant than the coliform bacteria to chlorine (4, 10, 16, 18, 20, 23). These reports and the incidence of diseases, such as hepatitis, giardiasis, and gastroenteritis, have periodically prompted reconsideration of the coliform bacteria as microbial indicators of water sanitary quality (17).

Levels of *L. pneumophila* ranging from 9×10^3 to 3.3×10^7 organisms per ml have been detected by direct immunofluorescence in sur-

TABLE 3. Survival of environmental and clinical *Legionella* isolates under standard conditions[a]

Bacteria	Source	% Legionellae surviving after following min of chlorine treatment:							
		1	5	10	30	60	90	120	150
L. pneumophila									
Serogroup 1	Allegheny River	65[b]	19	13	4	<1	<1	<1	<1
Serogroup 1	Hot water tank	56	19	20	17	6	<1	<1	<1
Serogroup 1	CDC[c] (Philadelphia 1)	85	20	11	10	5	3	<1	<1
Serogroup 6	Hot water tank	47	15	6	6	4	4	2	<1
L. micdadei	Hot water tank	31	9	6	4	3	2	<1	<1
	Clinical specimen	55	20	9	6	3	2	<1	<1

[a] Free residual chlorine, 0.1 mg/liter; temperature, 21°C; pH, 7.6.
[b] Compared with the concentration of legionellae before the addition of chlorine.
[c] CDC, Centers for Disease Control.

face waters (14). The recent detection of *L. pneumophila* in the plumbing systems of institutions has raised the suspicion that municipal drinking water systems serve as pathways for this contamination (9, 25). Our study directly involved a measurement of the effectiveness of chlorine in killing *L. pneumophila* and indirectly involved an assessment of the coliform bacteria as indicators of this process. Our results with *E. coli* are consistent with those of earlier workers: a 99% kill of these bacteria is achieved within a very short period of time. In contrast to these results, *L. pneumophila* may survive for periods of longer than 1 h under the same conditions. The bactericidal action of the chlorine is enhanced at higher temperatures and at lower pH levels. These findings are consistent with studies which were done with other bacteria (4, 5). Thus, the survival of *L. pneumophilia* in chlorinated waters may vary with the season and geographic area.

As stated previously, the criterion for a sanitary quality of water supplies is elimination of coliform bacteria. Our observation that legionellae are more resistant than coliform bacteria suggests the possibility that small numbers of legionellae may occasionally survive in waters that have been judged to be microbiologically acceptable. This difference in susceptibility to chlorine tends to increase as conditions become less optimal, e.g., higher pH, lower temperature, and lower chlorine concentration. These findings support the hypothesis that small numbers of legionellae may pass through public water supplies and subsequently contaminate internal plumbing systems. It should be noted that, to date, *L. pneumophilia* has not been isolated from water in reservoirs or in the water supply mains. Currently available methodology does not appear to be sufficiently sensitive to detect very low numbers of legionellae. Even if these bacteria are present in potable water, the extent of the hazard posed is not entirely clear. Plumbing systems and potable water have been shown to contain legionellae in some institutions in which outbreaks of Legionnaires disease were occurring (12, 25, 27). However, these bacteria have also been found in natural waters in the absence of any association with disease and in the plumbing systems of institutions in which no or only infrequent sporadic disease had been detected (14, 26, 29).

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant AI 17047 from the National Institute of Allergy and Infectious Diseases and sponsored by the Environmental Epidemiology Center of the Graduate School of Public Health of the University of Pittsburgh under the support of cooperative agreement CR80681-01-2 with the U.S. Environmental Protection Agency.

We thank Lorraine Mezmar, Ronald Speranza, and Randy Wolford for their technical assistance, Monto Ho for his encouragement, advice, and critical review of this manuscript, and Richard Cosentino for his encouragement.

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